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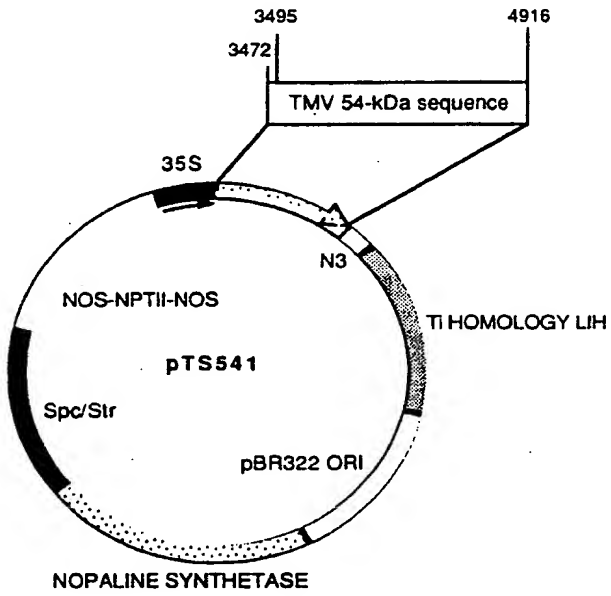

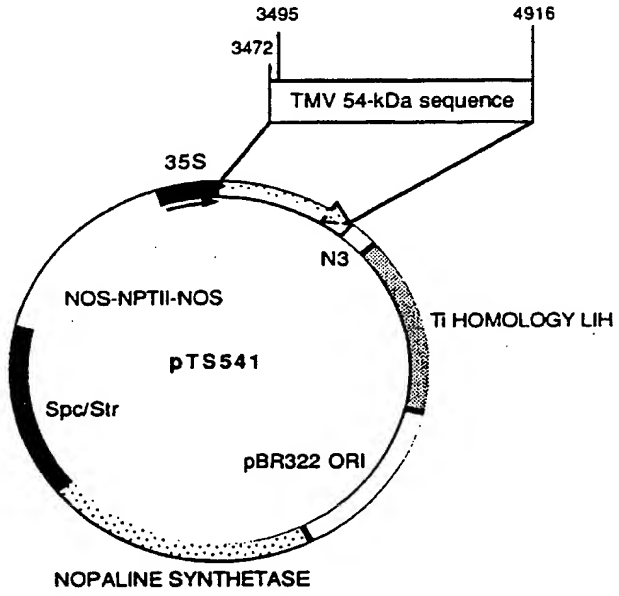

(57) Abstract

The present invention describes a method for inducing resistance to plant virus diseases by the transformation of plants susceptible to virus disease with a replicase portion taken from the plant virus genome; the resulting transformed plants, seeds and plants grown from the seeds exhibit resistance to disease brought about by the pathogenic virus from which the replicase portion of the virus genome was used for transformation.

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VIRAL RESISTANCE BY PLANT TRANSFORMATION WITH A REPLICASE PORTION OF A PLANT VIRUS GENOME

This is a Continuation-In-Part of our previously filed United States Patent Application 07/491,473, filed March 12th 1990.

Since the 1986 paper of P. Powell-Abel et al [see *Science* 223:738] showing that plants transformed with and expressing the coat protein gene of tobacco mosaic virus (TMV) are resistant to TMV, there have been a number of other examples of this concept which will undoubtedly have important implications for the protection of many crop species from various viral infections. To date, for example, viral coat protein-mediated resistance has been shown with at least 25 viruses in 15 taxonomic groups including alfalfa mosaic virus, tobacco rattle virus, potato virus X, cucumber mosaic virus (CMV), potyviruses, and plants transformed with both potato virus X and potato virus Y coat protein.

Plant virus sequences other than those coding for the viral coat protein have been tested to determine if transformed plants can be made to exhibit resistance to post-transformation viral infection. Positive sense sequences of alfalfa mosaic virus comprising almost full length copies of RNAs 1 and 2 failed to induce resistance in transformed plants [see Virology 163:572 (1988)]; anti-sense sequences of the TMV and potato virus coat protein genes did induce a low level of resistance in transformed tobacco [see Proc. Nat'l Acad. Sci., USA 86:6949 (1989); and EMBO Journal 7:1273 (1988)]; likewise antisense RNAs from one of three regions tested (5' sequences of RNA 1) of the CMV genome gave a low level of resistance in one transformant line.

Other forms of resistance using plant transformations with DNAs prepared from satellite RNAs of plant viruses have been reported, such as the use of the satellite of CMV [see Nature 328:799 (1987)] and the concept of the ribozyme based on sequences from satellite RNAs which possess the capacity to self cleave [see Nature 334:585 (1988)].

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The invention described herein represents an entirely new type of virus-induced resistance which may be transferred from one plant generation to another. The present invention discloses that transgenic plants containing a coding sequence, taken from all or part of the replicase portion of the viral genome, are resistant to subsequent disease by the virus; although there may be a very benign degree of virus synthesis in the inoculated leaf, the virus has been found not to spread and hence no disease develops. In the description which follows, the use of the 54 kDa coding sequence from TMV and a modified cDNA of RNA-2 which encodes one component of the polymerase of cucumber mosaic virus are described as two specific examples of the broader technology according to the present invention. Thus, in its broadest aspects, the present invention defines a means for bringing about viral resistance in plants which have been transformed with DNA copies of fragments or segments taken from the replicase portion of the pathogenic virus genome. In addition, the present invention defines transformed plants and their seeds which carry a portion of the viral genome which codes for a portion of the replicase genome of the pathogenic virus. According to the present invention, transformed plants that contain a portion of the viral replicase gene within their genome are resistant to subsequent viral disease from the virus from which the portion was derived, and these plants may also be resistant to subsequent disease from other closely related viruses.

In the exemplified tobacco mosaic virus description which follows, the presence of the 54 kDa sequence prevents the development of local chlorosis or necrosis and any systemic development of symptoms or virus replication associated with TMV infection.

The organization of the TMV genome is fairly well understood and accepted by the scientific community. Reading from the 5' towards the 3' end of TMV RNA, open reading frames code for 126- and 183 kDa proteins, a 30 kDa movement protein, and the 17.5 kDa coat protein. However, one aspect of the genome strategy that has not been fully elucidated is the exact nature of the replicase enzyme responsible for the synthesis of the genomic and subgenomic RNAs. While it is

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generally accepted that the virus codes for four proteins, two of which are coded for by the genomic RNA, and two of which are coded for by individual subgenomic RNAs, it is not generally accepted that the virus codes for at least one other additional and separate protein.

N. D. Young et al reported [see J. Cell Science Supplement 7:277 (1987)] that the 5'-proximal region of the genomic RNA which encodes two coinitiated proteins, the 126 kDa and 183 kDa proteins, are components of the replicase. The 183 kDa protein is generated by a read-through of the UAG stop codon of the 126 kDa protein. The other two proteins (with known functions), the 30 kDa protein and the coat protein are each synthesized from separate subgenomic mRNAs on which each gene is 5' proximal.

What is generally not accepted, however, is our contention that there is a separate protein, which we have labelled the 54 kDa protein, for which there is an open reading frame in the read-through portion of the 183 kDa gene. The principal evidence for the existence of this protein comes from the finding that there is a third subgenomic RNA in TMV infected plants, termed I_1 RNA, which initiates at nucleotide residue 3405 in the TMV genome and contains the open reading frame for a 54 kDa protein [see Virology 145:132 (1985)]. Support for its function as a mRNA and as a subgenomic RNA is derived from the observation that it is found on polyribosomes and that there is a double-stranded RNA of a size corresponding to the double-stranded version of the I_1 subgenomic RNA [see Virology 113:417 (1981), and Virology 131:533 (1983)].

More specifically, the following sequence of the region of the TMV genome containing the readthrough portion of the 183 kDa protein gene is:

```
    3405      3472                    3495          4919
5'—GCAGGA——CAAAGACUGGUGAUAUUUCUGAUAUG——AGUUGUUAA——3'
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This sequence depicts a portion of the I_1 subgenomic RNA beginning at nucleotide residue 3405 [the complete genome of TMV is 6,395 nucleotides long and may be found in Goelet et al, Proc. Natl. Acad. Sci USA 79:5818 (1982)]. The I_1 RNA terminates at nucleotide

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6395. In this sequence, the 54 kDa open reading frame according to the present invention extends from nucleotide residues 3495 to 4919, and the underlined region designates the sequence used for the plant transformation more fully described in the following examples.

More specifically, the gene portion for the 54 kDa protein within the I_1 RNA sequence is:

AUGCAG UUUUACUAUG AUAAGUGUCU CCCAGGCAAC AGCACCAUGA 46
UGAAUAAUUU UGAUGCUGUU ACCAUGAGGU UGACUGACAU UUCAUUGAAU 96
GUCAAAGAUU GCAUAUUGGA UAUGUCUAAG UCUGUUGCUG CGCCUAAGGA 146
UCAAAUCAAA CCACUAAUAC CUAUGGUACG AACGGCGGCA GAAAUGCCAC 196
GCCAGACUGG ACUAUUGGAA AAUUUAGUGG CGAUGAUUAA AAGGAACUUU 246
AACGCACCCG AGUUGUCUGG CAUCAUUGAU AUUGAAAAUA CUGCAUCUUU 296
AGUUGUAGAU AAGUUUUUUG AUAGUUAUUU GCUUAAAGAA AAAAGAAAAC 346
CAAAUAAAAA UGUUUCUUUG UUCAGUAGAG AGUCUCUCAA UAGAUGGUUA 396
GAAAAGCAGG AACAGGUAAC AAUAGGCCAG CUCGCAGAUU UUGAUUUUGU 446
AGAUUUGCCA GCAGUUGAUC AGUACAGACA CAUGAUUAAA GCACAACCCA 496
AGCAAAAAUU GGACACUUCA AUCCAAACGG AGUACCCGGC UUUGCAGACG 546
AUUGUGUACC AUUCAAAAAA GAUCAAUGCA AUAUUUGGCC CGUUGUUUAG 596
UGAGCUUACU AGGCAAUUAC UGGACAGUGU UGAUUCGAGC AGAUUUUUGU 646
UUUUCACAAG AAAGACACCA GCGCAGAUUG AGGAUUUCUU CGGAGAUCUC 696
GACAGUCAUG UGCCGAUGGA UGUCUUGGAG CUGGAUAUAU CAAAAUACGA 746
CAAAUCUCAG AAUGAAUUCC ACUGUGCAGU AGAAUACGAG AUCUGGCGAA 796
GAUUGGGUUU UGAAGACUUC UUGGGAGAAG UUUGGAAACA AGGGCAUAGA 846
AAGACCACCC UCAAGGAUUA UACCGCAGGU AUAAAAACUU GCAUCUGGUA 896
UCAAAGAAAG AGCGGGGACG UCACGACGUU CAUUGGAAAC ACUGUGAUCA 946
UUGCUGCAUG UUUGGCCUCG AUGCUUCCGA UGGAGAAAAU AAUCAAAGGA 996
GCCUUUUGCG GUGACGAUAG UCUGCUGUAC UUUCCAAAGG GUUGUGAGUU 1046
UCCGGAUGUG CAACACUCCG CGAAUCUUAU GUGGAAUUUU GAAGCAAAAC 1096
UGUUUAAAAA ACAGUAUGGA UACUUUUGCG GAAGAUAUGU AAUACAUCAC 1146
GACAGAGGAU GCAUUGUGUA UUACGAUCCC CUAAAGUUGA UCUCGAAACU 1196
UGGUGCUAAA CACAUCAAGG AUUGGGAACA CUUGGAGGAG UUCAGAAGGU 1246
CUCUUUGUGA UGUUGCUGUU UCGUUGAACA AUUGUGCGUA UUACACACAG 1296
UUGGACGACG CUGUAUGGGA GGUUCAUAAG ACCGCCCCUC CAGGUUCGUU 1346
UGUUUAUAAA AGUCUGGUGA AGUAUUUGUC UGAUAAAGUU CUUUUUAGAA 1396
GUUUGUUUAU AGAUGGCUCU AGUUGUUAA 1425

Unfortunately, the 54 kDa protein has not been found in infected tissues. When antibodies to a ß-galactosidase fusion protein for 432 amino acids specific to the read-through of the 126 kDa protein expressed in *Escherichia coli* were prepared, the 54 kDa protein in protoplast extracts could not be detected by either

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immunoprecipitation or Western blotting under conditions where the antibody would detect the 183 kDa protein [see T. Saito et al, Mol. Gen. Genet. 205:82 (1986)]. Likewise, the 54 kDa protein has not been detectable in Western blots using antiserum made to the whole protein [see G. J. Hills et al, *Virology* 158:488 (1987)], although on occasion faint bands in the region of the gel where such a protein would be expected have been seen. The antiserum made to the whole protein is, however, capable of precipitating the 54 kDa protein generated from *in vitro* translation products of either TMV RNA or T7 transcripts of the 54 kDa protein gene.

In an effort to attribute a function to the 54 kDa protein, we have transformed tobacco with the coding sequence for this nonstructural viral protein. Unexpectedly, these transformed plants show a complete resistance to replication in the uninoculated leaves of the U_1 strain of TMV from which the 54 kDa sequence was derived. This resistance was manifested when plants were inoculated with either high concentrations of virus or viral RNA.

In addition, the resistance exhibited by the 54-kDA transgenic plants differed in several important respects from TMV coat protein-mediated resistance: resistance was exhibited against both TMV RNA and TMV virions; it did not appear to break down over time or with increasing concentrations of innoculum; and it was effective against the TMV strain from which the 54 kDa protein gene was derived and a closely related mutant, but not against other TMV strains or other viruses.

Accordingly, a novel aspect of the present invention, is the conveyance of viral resistance to a plant which has previously undergone transformation of its normal genome with a portion of the replicase region of a viral genome, in its "sense" orientation.

A more complete understanding of this aspect, as well as others of the present invention. can be had by reference to the following figures and examples.

Figure 1 depicts plant expression vectors according to the present invention containing the TMV 54 kDa coding sequence inserted

between the CaMV 35S promoter and the nopaline synthase polyadenylation site;

Figure 2 depicts the plant expression vector containing the modified Fny-CMV RNA-2 gene sequence inserted between the CaMV 35S promoter and the nopaline synthase polyadenlyation site;

Figure 3 depicts the construction of a modified Fny-CMV RNA-2 chimeric gene for integration into the genome of a host plant;

More specifically, Figure 1 shows plasmids which were derived by insertion of the TMV cDNA into either the *Xho* I site or the *Sma* I site in the polylinker region of pMON316. The numbers in these vectors refer to nucleotides in the TMV genome. The NPTII gene confers a selectable kanamycin resistance marker to transformed plants.

Figure 2 shows the plant expression vector containing the modified Fny-CMV RNA-2 gene sequence according to the present invention inserted between the CaMV 35S promoter (35S) and the nopaline synthase polyadenlyation site (NOS polyA). This plasmid (pCMV N/B-23), as will be described in detail within the following specification, was derived by inserting the modified Fny-CMV RNA-2 gene according to the present invention into the *Bam*HI site of pROK2, a binary plant transformation vector [see Nature 321:446 (1986)]. This was accomplished by digesting pFny N/B-4 with *SphI* which cut this plasmid at a site 5' of the RNA-2 cDNA sequences. A *Bam*HI-*Sph*I adapter was ligated to this *Sph*I site and then digested with *Bam*HI which cuts at a site 3' of the RNA-2 sequences, thereby liberating the entire modified cDNA molecule. This 3 kb fragment was subcloned via standard techinques into the *Bam*HI site of pROK2 to generate pCMV N/B-23. Prior to plant transformation, this construct was transferred to *Agrobacterium tumefaciens* strain LBA-4404 by tri-patental mating [see Methods Enzymol. 118:627 (1986)] mediated by *E. coli* strain MM294-pRK2013. The trans-conjugates were selected by resistance to kanamycin and streptomycin at 50 and 125 µg/ml, respectively. The numbers in the figure refere to nucleotides in the Fny-CMV RNA-2 sequence; the neomycin phosophotransferase II (NPTII) gene confers a selectable kanamycin resistance marker on transformed plants; LB and

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RB = the left and right border of DNA transferred to the plant genome during Agrobacterium tumefaciens-mediated plant transformation; Ori = origin of replication.

Figure 3 shows the construction of pCMV N/B-23 being done in two stages. First a 94 basepair region was deleted from the full length cDNA clone of Fny-CMV RNA-2. Second, this deletion derivative was subcloned into pROK2, the plant transformation vector.

As described more completely in Example XII, the plasmid pFny206 containing the full length cDNA clone of Fny-CMV RNA-2 in pIBI-76, was digested with the restriction enzymes NcoI and BstEII. This DNA was then treated with the Klenow fragment of *E. coli* DNA polymerase 1 which acts as a DNA modification enzyme to obtain a blunt ended molecule. This blunt ended DNA was then ligated and transformed by standard methods into *E. coli* JM 101. The plasmid which resulted from this was labelled pFnyN/B-4. This plasmid was analyzed by various restriction enzyme digestions and DNA sequencing and was shown to have a 94 basepair deletion. This deletion also resulted in a change in the open reading frame such that this gene now encoded a truncated protein of approximately 75 kDa. Cloning also resulted in the retention of an AUG as a potential translation initiator 87 nucleotides upstream of the AUG in the RNA 2 gene resulting in potential translation of an additional 29 amino acids at the amino terminus of the protein.

As further described in Examples XII and XIII, this modified Fny-CMV RNA-2 gene contained in pN/B-4 was subcloned into plant transformation vector pROK2. To facilitate this subcloning, a *Bam*H1 site was added to the 5' end of this gene. pN/B-4 was digested with *Sph*1 and a *Bam*H1-*Sph*1 adaptor was ligated to this *Sph*1 site located at the 5' end of the gene. Following this ligation reaction, the pB/N-4 was digested with *Bam*H1 which released a fragment approximately 2960 basepairs in size containing *Bam*H1 cohesive ends at both the 5' and 3' terminals. It was this fragment which was ligated to the *Bam*H1 site in pROK2, and the resulting plasmid was named pCMV N/B-23. This

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plasmid was used to transfer this modified Fny-CMV RNA-2 gene into tobacco for further testing described beginning with Example XIII.

With specific regard to Figure 3, the plants are named in a code indicating which construct of Fny-CMV RNA-2 was transformed into the plant, what culture was used in a particular transformation, and the particular regenerated plant. "N/B" indicates which construct of Fny-CMV RNA-2 was transformed into a particular plant; in the case of N/B 1-8, the construct in this plant is from pCMV N/B-23 (see Figure 2). The numbers "1" or "2" indicates which of two culture tubes were used to transform tobacco with the N/B construct. The second number appearing in the designation simply indicates the particular plant number. For example, the numeral "8" in the above example simply indicates that this is the eighth regenerated plant obtained in this transformation experiment.

EXAMPLE I

culture and maintenance of plant and virus strains

TMV strain U_1 was purified from infected *N. tabacum* cv. Turkish Samsun plants as described by A. Asselin et al [see Virology 91:173 (1978)]. Virus RNA was isolated by phenol extraction and ethanol precipitation. *N. tabacum* cv. Xanthi nn was used as a TMV-susceptible, systemic host, and *N. tabacum* cv. Xanthi nc as a local lesion host. Plants were maintained in a greenhouse or in a growth chamber with a 14 hour per 24 hour light cycle and at 24°C.

EXAMPLE II

cloning of the 54 kDa gene

A clone of the TMV 54 kDa gene was obtained by using a 22 base oligonucleotide primer consisting of a *Bam*H1 site linked to the 5' end of a sequence complementary to base residues 4906 to 4923 of the TMV RNA sequence. First strand DNA was synthesized by M-MLV reverse transcriptase and was rendered double stranded by sequential treatment with reverse transcriptase and Klenow relying on loop-back synthesis [see T. Maniatis et al, Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, NY) (1982)]. The double-stranded cDNA was digested with *Bam*H1 and ligated into the *Bam*H1 site of M13mp18. The

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clones examined lacked the *Bam*H1 site provided by the primer. This resulted in the deletion of the 54 kDa termination codon and the extension of the 54 kDa protein at its C-terminus by five amino acids. The 54 kDa insert was removed by digestion with HaeII, treated with Klenow to blunt end the 3' overhang, and finally digested with PstI. The insert was ligated into PstI/*Sma*I digested pBS(-) resulting in plasmid pRTT-1 which contains the TMV sequence from nucleotide residues 3472 to 4914 of the TMV RNA sequence. The orientation of the insert was such that transcription from the T7 promoter gives (+) sense transcripts as depicted in Fig. 1.

Sequencing showed that all the clones examined contained the sequence from position 3332, but lacked the *Bam*H1 site provided by the primer. This resulted in the deletion of the 54-kDa termination codon and the extension of the 54-kDa protein at its C terminus by five amino acids derived from the vector M13mp18. The presence of an intact open reading frame was verified by insertion of the TMV sequence into a T7 transcription vector; the T7 transcript was synthesized and translated in a reticulocyte lysate system. In vitro translation yielded the desired 54 kDa product which confirmed that the AUG at position 3495 functions as an initiation codon. The product was verified as the desired 54 kDa protein by immunoprecipitation using 54 kDa antiserum.

The TMV 54 kDa sequence insert of pRTT-1 was removed by digestion with HindIII and SacI, made blunt-ended by treatment with Klenow, and ligated into either the *Sma*I or XhoI site of pMON316 [see S.G. Rogers et al, *Methods in Enzymology* 118:627 (1986)]. pMON316 contains a unique XhoI site in a polylinker region located between the cauliflower mosaic virus (CaMV) 35S promoter and the nopaline synthase 3'-untranslated region. A *Sma*I site is found in the polylinker region as well as within the Ti plasmid homologous region of pMON316. Plasmid pTS541A was generated by insertion of the TMV sequence into the *Sma*I site which resulted in the deletion of the nopaline synthase 3'-untranslated region and a portion of the Ti homology region. Insertion of the TMV sequence into the XhoI site resulted in the formation of pTS541. Clones containing the 54 kDa sequence in either

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sense or antisense orientation were characterized and isolated. Each construct was transferred to *Agrobacterium tumefaciens* GV3111 carrying pTiB6S3-SE by means of a triparental mating system [see R. T. Fraley et al, *Bio/Technology* 3:629 (1985)], and transconjugants were selected by resistance to kanamycin and streptomycin.

The 54 kDa coding sequence was subcloned into the plant expression vector pMON316 such that it is preceded by the CaMV 35S promoter and followed by the nopaline synthase 3' untranslated region as depicted in Figure 2. This construct was ultimately transferred into tobacco plants by *Agrobacterium tumefaciens*-mediated leaf disk transformation. Transformants were selected on the basis of kanamycin resistance and the production of nopaline synthase. Four transformed plants were generated with pTS541 and four other plants with pTS541A which lacks the 3' nopaline synthase untranslated region and a portion of the Ti homology region located immediately downstream from the 54 kDa open reading frame. This deletion did not interfere with integration of the chimeric TMV 54 kDa gene sequence into the plant genome. Progeny seed was collected from each self-fertilized plant. Additionally, plants were transformed with the chimeric TMV gene such that 54 kDa antisense RNAs were produced. Two independent antisense transformants were selected and regenerated into mature plants.

EXAMPLE III

plant transformation

Cut pieces of sterile, TMV susceptible, *Nicotiana tabacum* cv. Xanthi nn leaves were transformed by the modified *Agrobacterium tumefaciens* GV3111 containing the TMV 54 kDa coding sequence as described by Horsch [see Science 227:1229 (1985)]. Transformed calli were selected on regeneration medium supplemented with kanamycin at a concentration of 300 µg/ml. Resistant calli were induced to regenerate shoots and roots, transferred to soil, and maintained in a greenhouse.

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EXAMPLE IV

nucleic acid analysis

DNA was isolated from leaves of plants by a modified procedure of Murray and Thompson [see Nucleic Acids Research 8:4321 (1980)]. The DNA was digested with restriction enzymes, separated in 1.0% agarose gels, transferred to a nylon membrane, and hybridized to a ^{32}P-labeled probe specific for the TMV 54 kDa sequence. RNA was isolated from leaf tissue and total RNA was separated in a 1.2% agarose gel containing formaldehyde and transferred to nitrocellulose filter paper. The blot was hybridized to a ^{32}P-labeled probe complementary to the 54 kDa coding sequence. Six of the independently transformed plants were analyzed for expression of the chimeric gene. Genomic DNA was isolated from transformed and untransformed *N. tabacum* cv Xanthi nn. *Bam*H1 digests of the genomic DNA were hybridized to a ^{32}P-labeled TMV 54 kDa sequence specific probe. Hybridization to a 3.0 kb fragment verified the presence of a full length 54 kDa coding sequence. The 54 kDa sequence insert is 1.44 kb and another 1.59 kb is contributed by flanking vector DNA. The copy number of the 54 kDa protein gene in transgenic plants, as determined by Southern analysis, varied from 1 to 5 copies per diploid genome between different transgenic plants; no copies of the 54 kDa sequence were detected in nontransformed plants nor in plants transformed with pMON316 lacking the 54 kDa sequence insert.

The TMV 54 kDa transcripts extracted from transformed plants were also examined by Northern analysis for RNA. The expected size for the chimeric MRNA of 1.6 kb was identified in total RNA from each transgenic plant. Plants containing the integrate plasmid that lacks the 3' nopaline synthase untranslated region and the Ti homologous region also synthesize a 1.6 kb transcript. In addition, a larger transcript was synthesized which might result from the lack of the termination sequence usually contributed by the nos 3' sequence. In all plants, a number of smaller unidentified transcripts were also detected. Plants transformed with the vector alone did not produce any transcripts that hybridize with the the TMV 54 kDa sequence probe.

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The transgenic plants were also analyzed for expression of the TMV 54 kDa protein in accordance with Example IV. When analyzed using the Western blotting or immunoprecipitation procedures described, a 54 kDa protein could not be detected from the 54 kDa transgenic plants or from protoplasts prepared from 54 kDa transgenic plants or the controls.

EXAMPLE V

immunological analyses

An antiserum to the 54 kDa protein was made by injecting rabbits with a synthetic polypeptide representing an internal region, specifically amino acid residues 164 to 179, of the 54 kDa protein. An in vitro translation product of the 54 kDa T7 transcript was immunoprecipitable with the antiserum raised against the synthetic polypeptide. For western blotting, total extracts of the transformed and untransformed plants were prepared by homogenizing leaf samples in 50 mM Tris-HCl, pH 7.5, 1% sodium dodecyl sulfate (SDS), 10 mM 2-mercaptoethanol buffer; subjected to electrophoresis in a 12.5% SDS-polyacrylamide gel; and transferred to nitrocellulose filter paper. The filter was incubated first with specific antibodies followed by gold-conjugated anti-rabbit antibodies and silver enhancement.

In studies seeking the 54 kDa protein, 1-2 x 50 mm TMV-infected Turkish Samsun tobacco leaf strips were vacuum infiltrated with ^{35}S-methionine at a concentration of 10 µCi/ml in 10 mM KH_2PO_4 containing 1 mg/ml chloramphenicol. These were then incubated in dim light for 20 hrs at 25°C. Protoplasts were also labeled with ^{35}S-methionine. They were prepared from *Nicotiana tabacum* cv Xanthi NN leaves. The protoplasts (about 150,000/ml containing 5 to 10 µCi/ml of ^{35}S-methionine/ml) were incubated at 25°C in the light for 40 hours. These were then collected by low speed centrifugation, and lysed in 20 mM Tris-HCl, pH 7.5 buffer containing 2 mM EDTA, 0.5% SDS, 0.2% ß-mercapthethanol and 10 µg/ml phenylmethylsulphonyl fluoride as a protease inhibitor. Leaf strips were extracted in a mortar with a similar solution, but one which did not contain the inhibitor. The extracts were then clarified by microfuge centrifugation, and the

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supernatants examined for the 54 kDa protein. The presence of the 54 kDa protein was sought by incubating the extracts of the labeled leaves or protoplasts with antisera described above; an immunoprecipitation, polyacrylamide gel, and autoradiography assays were also conducted.

This antiserum was confirmed as being very active with in vitro translation products of the 54 kDa gene transcripts, and it could easily precipitate a 54 kDa protein from in vitro translation products of the RNA prepared from TMV virions containing the RNA necessary for manufacture of the 54 kDa protein. Protein could not be detected in leaves of either TMV infected plants or 54 kDa transformed plants.

EXAMPLE VI

inoculation of transformed plants

RI seedlings from self-fertilized transgenic plants were routinely inoculated with either 100 µg TMV-U_1 per ml of 50 mM phosphate buffer, pH 7.2, with Celite™ added as an abrasive, or TMV-U_1 RNA at a concentration of 300 µg/ml in pH 8.6, 50 mM Tris-phosphate buffer. Two leaves of each plant were inoculated. The volume of the inoculum was not standardized since inoculum concentration is the critical determinant as long as there is sufficient volume for adequate spread. In subsequent experiments, a closely related TMV mutant - mutant b6 as described by F. Garcia-Arenal et al, Virology 132:131 (1984) which is easier to score as a consequence of the bright yellow symptoms it elicits in the leaf. Plants were scored daily by visual observation of symptom development. In some instances, the presence of virus in inoculated plants was determined by probing leaf extracts with labeled cDNA to TMV.

In the first experiments to determine the susceptibility of the transgenic plants to infection by TMV, plants were inoculated with 50 µg TMV-U_1 per ml. Four rooted cuttings from each of the eight independently transformed plants containing the 54 kDa coding sequence, controls transformed with the vector alone, and several non-transformed Xanthi nn variants were inoculated. The plants were maintained in the greenhouse and monitored daily for symptom development. At 5 days post-inoculation, the transgenic controls and

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the non-transformed controls had clearly developed characteristic mosaic symptoms, while the transformed plants showed no sign of symptom development. No symptoms had developed on the transgenic plants by 48 days post-inoculation when the experiment was terminated. A homogenate of the inoculated and the upper leaves of those plants was used to inoculate the local lesion host, N. tabacum cv. Xanthi nc, to determine if a symptomless infection existed. No local lesions developed indicating the absence of detectable virus in these plants. All regenerated plants were resistant to TMV regardless of whether they were transformed with pTS541 which has the TMV sequence inserted into the complete pMON316 vector, or pTS541A which lacks the nos 3' untranslated region and the Ti homologous region. Plants transformed with the chimeric gene in the orientation which resulted in synthesis of the 54 kDa antisense RNA were not resistant to infection with TMV. However, these plants did demonstrate a delay in system development as compared to the vector transformed control. Since this was merely a delay in symptom development, these plants were not examined any further.

Progeny seedlings from self-fertilized transgenic plants were also analyzed for inheritability of the resistance phenomenon. R1 generation seeds were germinated on tissue culture medium containing 300 µg kanamycin per ml. Kanamycin-sensitive seedlings were considered to be those that were chlorotic and did not grow beyond the cotyledon stage. The segregation ratio of the seedlings expressing kanamycin resistance to those susceptible to kanamycin indicates that in each of the original transformants the NPTII gene was integrated at multiple loci. When seeds from self-fertilized transgenic plants were germinated on medium containing 300 µg kanamycin per ml, 95% of the seedlings emerged as being resistant to kanamycin and 5% of the seedlings became chlorotic. When transgenic seedlings were inoculated with TMV-U1 at a concentration of 100 µg/ml, 24% of these plants developed symptoms while the remaining 76 % demonstrated resistance to virus infection. Thus, the resistance to TMV segregated at approximately a 3:1 ratio (resistance:susceptible) whereas the

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seedlings had segregated at a ratio of approximately 19:1 with respect to the resistance to kanamycin. The large number of kanamycin resistant "escapees" makes this an unreliable means of screening progeny seedlings for expressors of the integrated chimeric TMV gene. All subsequent infection experiments were done with the segregating population of line 541A11 derived R1 seedlings.

In an experiment to determine the level of resistance, seedlings were inoculated with varying concentrations of TMV. Resistance was observed at concentrations up to 500 µg of TMV per ml. The resistant plants were maintained for 30 days post-inoculation without any subsequent development of symptoms. Leaf samples were taken from the inoculated plants to assay for virus replication and spread of virus. Extracts of the leaf samples were probed with cDNA prepared from purified TMV RNA. Virus could not be detected in either the inoculated leaves nor in the systemic leaves of the plants that demonstrated resistance, indicating that there is no viral replication in the resistant plants and that the resistance is absolute and not just a suppression of symptom development resulting in an asymptomatic spread of the virus throughout the plant. Transgenic plants containing the vector alone without the TMV sequence and nontransformed plants were used as controls, and virus was easily detectable in both types of control plants as well as in the progeny segregants that developed symptoms.

As a final evaluation of the resistance to viral infection of the transgenic plants, some plants were transferred immediately after inoculation to a growth chamber maintained at 31°C, to determine if the 54 kDa-induced resistance to TMV is temperature sensitive. Of the seven inoculated plants which carry the 54 kDa gene sequence, five did not develop symptoms at 31°C whereas all control plants developed symptoms typical to those kept at 24°C.

In conclusion, the preceding description has demonstrated the novel aspect of the present invention that transgenic plants containing a coding sequence portion of a viral genome associated with the replicase region of the virus are resistant to infection with the virus from which the portion was initially obtained.

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When compared with viral coat induced resistance, a number of advantages are present in the present invention. For example, the resistance to viral infection utilizing a replicase related coding sequence as described in the present invention is not as "fragile" as coat protein-induced resistance in which resistance breaks down when high concentrations of inoculum are used. In contrast, with the present invention, complete resistance is observed in non-inoculated leaves of plants challenged with high concentrations of virus or viral RNA. Whereas the protection mediated by the coat proteins of TMV and A1MV can be overcome by inoculating with viral RNA, the induced resistance according to the present invention utilizing the 54 kDa code sequence remains uncompromised when challenged with viral RNA. The level of resistance in 54 kDa transgenic plants does not appear to be due to the level of expression: plants with only one copy of the gene sequence did not show a decrease in resistance to intact virons or viral RNA. A single copy of the TMV coat protein is also sufficient to protect the plant whereas one copy of the AlMV coat protein is not.

In addition to the above, studies were also conducted which were aimed at discovering the stage at which the virus life cycle is disrupted using a portion of the replicase genome according to the present invention.

EXAMPLE VII

Tobacco plants (*Nicotiana tabacum* L), cv. *Xanthi* nn, as well as the 54 kDa transgenic *Xanthi* nn, and the TMV local lesion indicator host *Xanthi* NN, were maintained under greenhouse conditions. Plants used for protoplast preparation were transferred to a growth chamber on a 14 hr light/10 hr dark cycle at 24°C for at least one week prior to use. The light intensity was reduced to 125-150 $\mu E \cdot m^{-2} \cdot s^{-1}$ by shading with cheese cloth. TMV strains U1 and U2 [see Phytopathology 44:277 (1954)] were purified [see Virology 91:133 (1978)]. The TMV strain U1 used in some of the following examples was derived from transcripts generated from a full length cDNA clone of the virus. Virus infection of whole plants was achieved by inoculation of both upper and lower surfaces of fully expanded *Xanthi* nn or 54 kDa transgenic tobacco

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leaves with 0.05, 0.5. or 1.0 mg/ml of TMV strain U1 in 0.05 M potassium phosphate, pH 7.0 buffer, with Celite as an abrasive. Viral RNA was prepared from TMV strains U1 and U2 by phenol extraction and ethanol precipitation.

EXAMPLE VIII

protoplast preparation and infection

Protoplasts were obtained from leaves of 54 kDa transgenic plants and control, nontransgenic tobacco plants. The protoplasts (0.5-1.0 x 10^6 cells/ml) were infected by electroporation with viral RNA extracted from TMV strains U1 or U2. Electroporation was performed in a final volume of 2 ml of sterile 0.7 ml of sterile 0.7 M mannitol, using a single ring electrode (2.5 mm high, 1 cm gap) connected to a ProGenetor 1 electroporation apparatus by applying two 5 msec pulses of 300 V. The viral RNA concentrations ranged from 10 to 100 µg/ml although routinely 10 µg/ml was used. In addition, all experiments included a set of mock-inoculated protoplasts electroporated in buffer alone.

After electroporation, protoplasts were resuspended in incubation medium: 0.7 M mannitol containing 1 mM KNO_3, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1µM KI, 0.01 µM $CuSO_4$, 10 µg/ml rimocidin, and 100 µg/ml carbenicillin buffered with 50 mM citrate, pH 5.5 buffer. The protoplasts (3 ml) were transferred to agar plates (1% noble agar in incubation medium prepared in 60- x 15-mm petri dishes) and incubated in low light at 25°C [see Virology 161:488 (1987)].

EXAMPLE IX

analysis of protoplast proteins

Accumulation of TMV coat protein in protoplasts was detected by western blotting. Protoplasts were harvested by low speed centrifugation and disrupted in 50-100 µl of SDS - polyacrylamide gel electrophoresis (PAGE) sample buffer [see Nature 227:680].

The released proteins were separated by SDS-PAGE, electoblotted to nitrocellulose, and probed using a rabbit polyclonal antiserum (diluted 1:1,000) to strain U1 TMV coat protein and [^{125}I] protein A. To monitor the synthesis of virus-encoded proteins in protoplasts, L-[^{35}S]

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methionine was added to the incubation medium at a concentration of 10 µCi/ml. After continuous labeling, protoplasts were washed in 0.7 ml mannitol and disrupted in buffer. [^{35}S]-labeled proteins were analyzed by SDS-PAGE [see Nature 227:680 (1970)] and autoradiography.

EXAMPLE X

analysis of RNA

At various times after electroporation, protoplasts were harvested, washed in sterile 0.7 M mannitol, disrupted in 50 mM Tris HCl, pH 8.0 buffer, 10 mM EDTA, 2% SDS, and extracted with phenol/chloroform/isoamyl alcohol (50:50:1). In some instances, following ethanol precipitation, lithium chloride-soluble (enriched in ds RNA) and lithium chloride-insoluble (enriched in ss RNA) fractions were prepared [see Mol. Cell. Bio. 5:2238 (1985)]. Leaf RNA was prepared in the same manner starting with leaf tissue pulverized in liquid nitrogen.

RNAs were separated on formaldehyde-containing, 1.2% agarose gels and were blotted to nitrocellulose, which was then probed with in vitro-synthesized, [^{32}P]-labeled, ssRNA transcripts. Northern blots were prehybridized and hybridized for 24 hrs at 45°C in 5X SSC (1X SSC = 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0), 5X Denhardt's solution, 50 mM sodium phosphate, pH 7.0, 0.1% SDS, 250 µg/ml of yeast RNA, and 50% formamide, and were washed five times in 0.1X SSC, 0.2% SDS at 65°C. Relative amounts of specifically hybridizing RNA bands were qualified by excising the appropriate areas of the nitrocellulose filter using an autoradiograph as a template and determining the amount of radioactive probe bound using a liquid scintillation spectrometer.

In vitro-synthesized RNA probes were prepared from two DNA templates: 1) T3 polymerase transcription [see Molecular Cloning, Cold Spring Harbor Laboratories (1989)] of pBS126, a derivative of pBSM13- containing an insert corresponding to nucleotides 1-3,785 of strain U1 TMV, including the whole of the 126 kDa protein reading frame, yields a (+) sense transcript corresponding to this region of TMV genomic RNA and complementary to the 3' region of full length (-) sense TMV RNA; 2)

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SP6 polymerase transcription [see Molecular Cloning, Cold Spring Harbor Laboratories (1989)] of pSP64 derivative containing an insert corresponding to the coat protein gene of TMV (from nucleotide 5,663 to the 3' end). This yields a (-) sense transcript complementary to the (+) sense, full length, as well as the subgenomic TMV RNAs, all of which possess the same 3' terminus. Additionally, T7 transcripts of pRTT-1, containing the sequence encoding the strain U1 TMV 54 kDa protein were prepared and used to program wheat-germ [see PNAS (USA)70:2330 (1973)] and reticulocyte lysate-derived [see Eur. J. Biochem. 67:247 (1976)], in vitro translation systems.

The protoplasts from the 54 kDa transgenic plants (see Example VIII) that were electroporated 24 or 48 hrs earlier with strain UI TMV RNA did not contain any infectious virus detectable by bioassay on local lesion indicator plants, but under the same experimental conditions, these protoplasts replicated infectious strain U2 TMV. In contrast, control protoplasts from nontransformed plants replicated both strains of TMV. According to bioassay data, protoplasts derived from 54 kDa transgenic plants remained resistant to strain U1 TMV RNA even when the inoculum concentration was increased from 10 to 100 µg/ml of RNA. Consistent with bioassay data, western blot analysis of protoplast proteins showed that 54 kDa transgenic protoplasts accumulated no detectable strain U1 TMV coat protein, although under the same conditions these cells accumulated strain U2 TMV coat protein in amounts similar to those in the control, nontransgenic tobacco protoplasts.

These results indicate that the resistance displayed by whole, intact 54 kDa transgenic plants according to the present invention is retained by protoplasts prepared from them, and that the resistance mechanism functions at the level of the single cell. This implies that resistance at the whole plant level is not due primarily to a block in cell-to-cell or long distance virus spread, but must act either by preventing the initiation of virus infection or by inhibiting virus replication once infection has taken place. This conclusion is consistent with data showing that plasmodesmata (the routes of cell to

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cell virus spread) in 54 kDa transgenic plants appear to be unmodified and have normal molecular exclusion limits.

The 126 kDa protein is the more abundant of the two known viral-coded TMV replicase components and its synthesis, directed by the 5' proximal open reading frame of TMV genomic RNA is probably the first step in replication after (or during) virus uncoating. The 126 kDa protein was not apparent among [^{35}S]-labeled proteins extracted from 54 kDa transgenic protoplasts infected with strain U1 TMV. However, under the same conditions the 126 kDa protein was present in extracts of [^{35}S]-labeled protein from nontransgenic tobacco protoplasts infected with strain U1 TMV. The equivalent, faster moving protein encoded by strain U2 TMV was synthesized in both transgenic and nontransgenic protoplasts infected with that strain of TMV. Similarly, synthesis of strain U2 TMV coat protein was observed in both cell types. Synthesis of strain U1 TMV coat protein could not be observed in this way because it lacks methionine. Attempts to improve the sensitivity of detection of the 126- and 183 kDa proteins by immunoprecipitation with appropriate antisera were unsuccessful.

Although direct methods were not successful to demonstrate the synthesis of viral-coded replicase proteins in 54 kDa transgenic tobacco protoplasts infected with strain U1 TMV, there remained an indirect way of detecting the presence of these proteins. Specifically, any products of replicase activity will betray the presence of small levels of all of the replicase components. Because the initial product of replication is (-) sense RNA generated from the input, (+) sense, genomic TMV RNA, RNA from strain U1 TMV-infected 54 kDa transgenic tobacco protoplasts was probed for the presence of full length (-) sense TMV RNA. By 21 hrs post-inoculation, trace amounts of ss, (-) sense, full length TMV RNA were found to be present in the strain U1 TMV-infected 54 kDa transgenic tobacco protoplasts, although its ds form was not detectable. Therefore, some small quantity of virus-coded replicase components must have been synthesized after infection and must have been functional to some extent in these cells.

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Detection of (-) sense TMV RNA in the 54 kDa transgenic tobacco protoplasts prompted the study to determine if replication proceeded beyond (-) strand synthesis and resulted in any (+) strand synthesis. Northern analysis of protoplast RNAs with a probe specific for (+) sense, 3' sequences of TMV RNA detected the presence of low levels of (+) sense TMV RNAs by 5 hours post-inoculation and the full complement of full length and subgenomic TMV RNAs by 21 hours post-inoculation in strain U1 TMV-infected 54 kDa transgenic tobacco protoplasts. The full complement of ds forms of the full length and subgenomic TMV RNAs were also observed in the TMV-infected nontransgenic protoplasts. However, northern blot analysis was not sensitive enough to detect TMV dsRNAs in the TMV-infected 54 kDa transgenic protoplasts. Counting of the radioactive probe bound to specific ssRNA bands showed that the levels of full length (+) sense TMV RNAs which accumulated in strain U1 TMV-infected 54 kDa transgenic tobacco protoplasts, were between 20- and 80-fold less than those in infected nontransgenic protoplasts depending on the specific experiment. Similar results were obtained either the laboratory U1 strain TMV RNA or clone-derived U1 TMV RNA were used. The results indicate, therefore, that a low level of strain U1 TMV replication can occur in 54 kDa transgenic tobacco protoplasts.

The studies described in the above examples, particularly Examples VII - X, of 54 kDa transgenic tobacco protoplasts with strain U1 TMV indicated that these cells permit the synthesis of trace amounts of TMV-specific RNAs. Next examined was whether the results obtained with protoplasts truly reflected the characteristics of the resistance phenomenon in leaf cells of whole 54 kDa transgenic plants. To do this, leaves of 54 kDa transgenic tobacco plants were inoculated on their upper and lower surfaces with strain U1 TMV particles at concentrations of 0.05 and 0.5 mg/ml. These highly concentrated inocula (two and three orders of magnitude greater than those typically employed to infect nontransgenic tobacco plants) were used to maximize the number of leaf cells infected and thus increase the chances of detecting virus-specific RNAs.

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Northern blot analysis of RNA from 54 kDa transgenic tobacco leaves inoculated with strain U1 TMV at 0.05 mg/ml did not reveal any viral RNAs. However, increasing the inoculum to 0.5 mg/ml resulted in the production of detectable levels of full length and sub-genomic (+) sense TMV RNAs, which increased over time. Once again, any TMV double-stranded RNAs that may have been present were at levels too low to detect using this methodology. Comparison of the amounts of radioactive probe bound to specific RNA bands in Northern blots indicated that the levels of full length (+) sense TMV RNAs that accumulate in heavily inoculated 54 kDa transgenic tobacco leaves were 17- to 20-fold less than those found in nontransgenic tobacco tissue. Similar results were obtained both with laboratory virus isolate and with virus propagated from an infectious TMV cDNA clone (applied at a concentration of 1 mg/ml). When the heavily inoculated areas of 54 kDa transgenic tobacco leaves were used as sources of inoculum for assay on local lesion indicator plants, small amounts of biologically active virus were sometimes detected. No virus was detectable in other leaves of the same plant or on uninoculated parts of the same leaf when clone-derived virus was used as the inoculum.

Overall, the results obtained with heavily inoculated 54 kDa transgenic tobacco leaves appear consistent with those obtained using protoplasts in that there appears to be a major inhibition, although not a complete shutdown, of virus replication.

There are two possibilities to describe how the inhibition of replication of strain U1 TMV is achieved in 54 kDa transgenic tobacco plants according to the present invention. First, the 54 kDa protein or its RNA might directly inhibit replicase activity, or second, the 54 kDa protein or its RNA may act indirectly, for instance by inhibiting synthesis of the virus-coded replicase components, the 126- and 183 kDa proteins. The second possibility was addressed by translating TMV RNA in rabbit reticulocyte or wheat-germ in vitro translation systems that had also been programmed or preprogrammed with an in vitro-synthesized TNA transcript encoding the 54 kDa protein. In both of these cell-free translation systems, synthesis of the 126- and 183-, as

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well as the 54 kDa proteins occurred with no suggestion of specific inhibition of 126- or 183 kDa protein synthesis. Thus, there is no evidence that the 54 kDa protein or its RNA according to the present invention, affect the synthesis of virus-coded replicase components. The results are, therefore, consistent with the first possibility, namely that the 54 kDa protein or its corresponding RNA affect replicase activity directly.

In addition to TMV, studies were also conducted using cucumber mosaic virus (CMV) with analogous results. In these experiments, *Nicotiana tabacum* cv. Turkish Samsun NN plants were transformed with a modified cDNA clone of RNA-2, which encodes a transcript of the replicase component of CMV subgroup I strain Fny. The gene had been modified by deleting a 94 bp region spanning nucleotides 1857 to 1950 (Figure 2). More specifically, the partial nucleotide sequence of the Fny-CMV RNA-2 cDNA clone which was modified as described in Example XII and which contains the NcoI and BstEII sites used to generate a 94 basepair deletion is:

AAU ACC AUC GUC ACC AUG GCU GAG UUU GCC UGG UGU UAU GAC 42
ACC GAC CAA UUC GAA AAG CUU UUA UUC UCA GGC GAU GAU UCU 84
CUA GGA UUU UCA CUG CUU CCC CCU GUU GGU GAC CCG AGU AAA 126
UUC ACA 132

in which the first underlined portion corresponds to the *Nco* I restriction site and the bold "G" corresponds to nucleotide 1856, and in which the second underlined portion corresponds to the Bst EII restriction site and the bold "G" corresponds to nucleotide 1951; and the amino acid sequence is:

Asn Thr Ile Val Thr Met Ala Glu Phe Ala Trp Cys Tyr Asp Thr
5 10 15
Asp Gln Phe Glu Lys Leu Leu Phe Ser Gly Asp Asp Ser Leu Gly
20 25 30
Phe Ser Leu Leu Pro Pro Val Gly Asp Pro Ser Lys Phe Thr
35 40

Once the 94 nucleotide deletion has been made as described in Example XII, the nucleotide and amino acid sequence of the carboxy-terminus of the modified Fny-cDNA clone is:

ACC AUC GUC ACC AUG GUG ACC CGA GUA AAU UCA CAA CUC UUU 42

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UCA ACA UGG AAG CUA AGG UGA 63

in which the underlined portion corresponds to the prior underlined restriction sites and the bold "G"'s represent original nucleotides 1856 and 1951; and the amino acid sequence is:

Thr Ile Val Thr Met Val Thr Arg Val Asn Ser Gln Leu Phe Ser
5 10 15
Thr Trp Lys Leu Arg
20

The region surrounding and including this 94 bp region contains four domains which are highly conserved among putative replicase encoding sequences in many positive-sense RNA plant and animal viruses. This deleted 94 bp region also contained the third domain, the Gly-Asp-Asp domain, which has been shown to be necessary for replication of the bacteriophage Qß. As noted in the sequence above, this deletion also caused a shift in the open reading frame resulting in a truncated translation product. R_1 generation plants from the original transformants were tested for resistance to virus infection. Individual plants from 2 out of 18 transformed lines were resistant to systemic disease when inoculated with either Fny-CMV virions or RNA at concentrations as high as 500 µg/ml. Resistance was also shown when plants were inoculated with viruliferous aphids, and was maintained when plants were challenged with the closely related subgroup I strains O, Sny Y, and Ve85, but not when challenged by LS, a subgroup II strain.

EXAMPLE XI

cucumber mosaic virus strains

Cucumber mosaic virus strains Fny, O, Sny, Ve85, Y, and LS were purified from *Nicotiana tabacum* cv. Turkish Samsun as described by Palukaitis [see Methods For Plant Biology, Weissbach and Weissbach eds., Academic Press, New York (1988)]. Viral RNA was isolated from intact cucumber mosaic virus virions by phenol/chloroform extraction and ethanol precipitation. Plants were routinely inoculated with virus in 50 mM sodium phosphate buffer, pH 7.2, or RNA in 50 mM Tris phosphate buffer, pH 8.9, with the use of the abrasive Celite. *Nicotiana tabacum* cv. Turkish Samsun NN was used for plant transformation and as a cucumber mosaic virus-susceptible systemic host. Plants were

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maintained in a green house or in a growth chamber at 24°C with a 16 hr/8 hr light-dark cycle.

EXAMPLE XII

modification and subcloning of cucumber mosaic virus RNA-2

The Fny-CMV cDNA clone pFny206 [see J Gen. Virol. 69:1777 (1988)], which encodes a full length non-infectious RNA-1, was modified and subcloned into a binary plant transformation vector as shown in Figure 2. This RNA-2 clone was modified by digesting pFny206 with the restriction endonuclease NcoI, phenol/chloroform extracted to remove these enzymes, and ethanol precipitated. This DNA was treated with Klenow fragment in the absence of nucleotides in an attempt to obtain a blunt-ended molecule. This DNA was then digested with the restriction endonuclease BstEII, treated with Klenow fragment in the presence of nucleotides to obtain a blunt ended molecule, phenol/chloroform extracted, and ethanol precipitated. This linear molecule was then recircularized by T4 DNA ligase to generate plasmid (pFnyN/B-4) which contained a cDNA clone of Fny-CMV RNA-2 in which 94 nucleotides from nucleotide 1857 to nucleotide 1950, inclusive, were deleted. This 94 nucleotide region contained the Gly-Asp-Asp domain that is highly conserved among replicase proteins of many positive-sense viruses. This deletion also caused a shift in the open reading frame which resulted in an in-frame translational stop codon 15 codons downstream of the deletion site. This deletion, therefore, not only deleted a 94 bp region, but also resulted in a truncated open reading frame. Cloning also resulted in the retention of an AUG as a potential translation initiator 87 nucleotides upstream of the AUG in the RNA 2 gene resulting in potential translation of an additional 29 amino acids at the amino terminus of the protein. The protein encoded by this modified gene is thus approximately 75 kDA in size compared with a wildtype protein of 96.7 kDa.

To subclone this modified Fny-CMV RNA-2 cDNA clone into a binary plant transformation vector, pN/B-4 was digested with *Sph*1 which cut this plasmid at a site 5' of the N-terminus of the RNA-2 cDNA deletion clone. An adapter containing a 5' *Bam*H1 overhang and a 3' *Sph*1

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overhang was ligated to this site and then digested with *Bam*H1 which cuts at a site of 3' of the C-terminus, thereby liberating the entire modified cDNA molecule. This 3kb fragment was subcloned via standard techniques into the *Bam*HI site of the binary plant transformation expression vector pRok2 [see Nature 321:446 (1986)], to generate pCMV N/B-23. This construct was transferred to *Agrobacterium tumefaciens* strain LBA-4404 by tri-parental mating [see Methods Enzymol. 118:627 (1986)] mediated by *E. coli* strain MM294-pRK2013. The transconjugates were selected by kanamycin and streptomycin at 50 and 125 ug/ml respectively. The nucleotide sequence of Fny-CMV RNA-2 in pFny206 and pCMV N/B-23 are shown in figure 2.

EXAMPLE XIII

plant transformation

Transformation of *Nicotiana tabacum* cv. Turkish Samsun NN was accomplished via *Agrobacterium fumefaciens*-mediated leaf disk transformation described by Horsch as in Example III. Two days after co-cultivation of the leaf discs with pCMV N/B-23 the leaf discs were transferred to shoot regeneration medium containing 500 µg/ml of carbenicillin, 300µg/ml of kanamycin, 1 mg/l 6-benzyl aminopurine (BAP), and 0.1 mg/l napthyl acid (NAA). Leaf discs growing in this medium were transferred to fresh medium every 7 to 14 days. By approximately 4 weeks, shoots formed which were transferred to root-inducing medium containing 300 µg/ml of Kanamycin, 0.2 mg/l BAP, and 0.1 mg/l of NAA. These shoots formed roots in approximately 2-3 weeks at which time they were transferred to vermiculite for 2 weeks, repotted to soil, and transferred to a growth chamber. Eighteen independent kanamycin resistant transformants were chosen for further analysis.

EXAMPLE XIV

assessment of resistance

Eighteen independent transformants were initially screened by a detached leaf assay for resistance to Fny-CMV infection. A leaf was removed from each RO generation transgenic plant and inoculated on the upper and lower epidermis with 50 µg/ml of Fny-CMV. The leaf stems

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were immersed in water and the leaves were incubated for 6 days at 25°C after which a leaf disc 2 cm in diameter was removed. Extracts of this tissue were probed with ^{32}P-labeled cDNA to Fny-CMV to determine if viral RNA was present (Palukaitis, 1988). The absence or presence of viral RNA indicated resistance or susceptibility to virus infection, respectively. These transgenic plants were allowed to flower and seeds of the R1 generation were collected and planted.

This assay indicated that seven of these transgenic lines were resistant to Fny-CMV infection, and the R1 generation seedlings from each of the 7 transformed lines which appeared resistant by the detached leaf assay. (N/B 1-1, 1-2, 1-8, 2-3, 2-5, 2-6 and 2-7) as well as one transgenic line (N/B 2-1) which did not show resistance in the detached leaf assay and non-transformed *N. tabacum* cv. Turkish Samsun NN plants were inoculated with 0.2, 1.0, 5, 10 and 100ug/ml of Fny-CMV. All of the putative resistant lines were either resistant or had a delay in symptoms at inoculum doses between 0.2 and 1.0 µg/ml as depicted in the following Table 1 which provides an assessment of replicase-mediated resistance to CMV infection in R1 generation transgenic tobacco plants:

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TABLE 1

Line[a]	Inoculum Concentration[b] (µg/ml) 0.2	1	5	10	100	500	20[f]	50[g]
N/B 1-1	4/12[c]	12/12	12/12	10/10	9/10	ND	ND	ND
N/B 1-2	1/12	13/34	2/12	17/33	16/36	21/26	8/10	ND
N/B 1-8	3/12	3/27	4/12	10/27	11/31	14/32	2/10	5/14
N/B 2-3	ND[e]	12/23	ND	23/25	26/26	25/25	ND	ND
N/B 2-5	6/12	10/11	12/12	10/10	10/10	ND	ND	ND
N/B 2-6	4/12	7/12	11/11	10/10	10/10	ND	ND	ND
N/B 2-7	10/12	10/12	11/11	10/10	10/10	ND	ND	ND
N/B 2-1	12/12	31/31	11/11	28/28	26/26	27/27	ND	ND
N/N	3/3	4/4	4/4	ND	ND	3/3	4/4	4/4

In the above Table 1,

[a] A preliminary detached leaf assay had indicated that all of these lines (except line N/B 2-1 and N/N) were resistant to Fny-CMV infection;

[b] Plants were inoculated with Fny-CMV in 50 mM $NaPO_4$ buffer, pH 7.2, with Celite as an abrasive. These data represent the number of plants showing symptoms at two weeks post inoculation as a function of the total number of inoculated plants;

[c] These numbers indicate the number of plants showing systemic symptoms over the total number of plants inoculated;

[e] ND = not determined;

[f] To determine if temperature affected resistance, plants were inoculated with Fny-CMV at 20 µg/ml and incubated at 30°C for two weeks; and

[g] Plants were inoculated with Fny-CMV RNA at 50 µg/ml in 10 mM Tris-HCl buffer, pH 8.9.

Two lines (N/B 1-2, N/B 1-8) in which the individual plants were resistant to these virus concentrations were tested for resistance to Fny-CMV inoculum concentrations of 500 µg/ml of virus and line N/B 1-8 was inoculated with 50 µg/ml of Fny-CMV RNA. Not all R1 generation plants from these lines were resistant due to Mendelian segregation of this resistance trait within these R1 populations. Plants were scored daily by visual observation of symptom development. In some cases the presence of viral RNA in these inoculated plants was determined by probing leaf extracts with ^{32}P-labelled Fny-CMV cDNA. R1 seedlings of lines N/B 1-2, 1-8, 2-3, and the two control lines were inoculated with Fny-CMV at 500 µg, and as shown in Table 1, 14 of 32 inoculated N/B 1-8 plants and 21 of 26 inoculated N/B 1-2 plants did develop symptoms of disease. Hence, it appears that very high inoculum doses can overwhelm some plants which might be resistant to lower inoculum doses. This presumption was confirmed in experiments with R2 generation plants where a few plants (3 of 35) which were resistant at 100 µg/ml showed symptoms when superinfected at 500 µg/ml. However neither viral RNA nor progeny virus could be detected in the uninoculated leaves of resistant plants using dot blot hybridization and bioassay, an indication that these plants exhibited true resistance and not merely symptom suppression.

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R_1 generation seedlings from two lines (N/B 1-2 and N/B 1-8), a transgenic line (N/B 2-1) which did not show resistance in the detached leaf assay, and non-transformed *Nicotiana tabacum* cv. Turkish Samsun NN plants were also tested for resistance to Fny-CMV infection by viruliferous aphids (*Myzus persicae*). *Nicotiana clevlandii* systemically infected with Fny-CMV acted as the virus source plants on which these aphids fed prior to feeding on the experimental plants. Following a 30 minute feeding period on the source plants these aphids were transferred to the transgenic and control plants with each plant receiving 10 aphids. The aphids were allowed to feed for approximately 15 hr after which the plants were fumigated with insecticide to kill the aphids. The resultant plants were resistant to Fny infection when transmitted by aphids.

EXAMPLE XV

genomic DNA analysis

Genomic DNA was isolated from leaf tissue by a modified procedure of Murray and Thompson (1980). High molecular weight DNA was digested with restriction enzymes, separated on a 0.8% agarose gel, transferred to GeneScreenPlus (Dupont) nylon membranes. This membrane was hybridized in 5x SSC, 5x Denhardts, 5% dextran sulfate and 2% SDS to a ^{32}P-labeled DNA probe specific for Fny-CMV RNA-2 gene sequence. All DNA probes were prepared by the random hexamer primer reaction [see Anal. Biochem 132:6 (1983)] to specific activities of at least 5×10^8 cpm/µg of DNA.

Genomic DNA from these 7 transgenic lines and line N/B 2-1 and the control line (ROK9) transformed with the pROK2 vector alone were examined as described. Southern Blot Hybridization analysis indicated that these 7 lines contained approximately 1 to 7 copies per haploid genome. The lines with the highest copy number, lines N/B 1-2 and 1-8, contained approximately 3-5 and 5-7 copies respectively. Line N/B 2-1, which is not resistant in the detached leaf assay, contained 1 copy per haploid genome. The control ROK9 line did not contain any Fny-CMV RNA-2 homologous sequences.

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To determine if this resistance was temperature sensitive, R1 seedlings of the resistant lines N/B 1-2 and 1-8, and control lines N/B 2-1 and untransformed *Nicotiana tabacum* cv. Turkish Samsun NN were inoculated with Fny-CMV at 20 µg/ml and immediately placed in a growth chamber maintained at 30°C. Within 4 days after inoculation all of the control plants developed systemic symptoms whereas many N/B 1-2 and 1-8 resistant plants were obtained, indicating that this resistance was not temperature sensitive (see Table 1).

Prior studies [see EMBO J. 6:1845 (1987); Bio/Technology 8:127 (1990), and Virology 159:299 (1987)] have shown that coat protein mediated protection of TMV and alfalfa mosaic virus can sometimes be overcome by inoculation with viral RNA. Therefore, R1 seedlings of line N/B 1-8, the non-resistant N/B 1-2 control line, and non-transformed *Nicotiana tabacum* cv. Turkish Samsun NN line were inoculated with 50 µg/ml of Fny-CMV RNA. While the control lines developed systemic symptoms in 100% of the plants by 4 days, only 36% of the segregating N/B 1-8 plants developed symptoms even after 14 days (Table 1). Is is apparent from these data that lines N/B 1-2 and 1-8 are resistant to both virus and viral RNA at very high inoculum levels.

The specificity of resistance was determined by challenging R1 seedlings of the resistant lines N/B 1-2 and the untransformed *Nicotiana tabacum* cv. Turkish Samsun NN with 20 µg/ml of three subgroup I strains (O, Sny, Y, Ve85) and one subgroup II strain (LS). The control lines developed symptoms in 100% of the plants by 7 days after inoculation will all four virus strains tested. Resistant N/B 1-2 and 1-8 plants were obtained when challenged with the three subgroup I strains but not when challenged with the subgroup II strain. This indicates that the resistance obtained as a result of practicing the present invention and obtaining transformed plants with a modified chimeric Fny-CMV RNA-2 gene provides resistance to infection by group I CMV virus strains.

The level of resistance appears to be affected by the number of copies of the defective CMV replicase gene inserted into the genome. The copy number of the original transformed plants tested in the

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detached leaf assay, ranged from 1-3 copies per haploid genome for the five lines which were not resistant, to 3-5 copies and 5-7 copies for the resistant N/B 1-2 and 1-8 lines, respectively. These two resistant lines also showed a difference in the number of resistant R1 plants at various inoculum concentrations (Table 1), which again suggests that the level of resistance of any given plant, particularly at very high inoculum concentrations of 100 to 500 µg/ml, may be affected by the number of copies of the modified RNA-2 Fny-CMV RNA 2 sequence present in the genome of that plant.

To assess the possible role the copy number may have in determining the level of resistance, both non-resistant and resistant R1 generation N/B 1-2 and 1-8 plants were analyzed. The presence of virus in uninoculated leaves, copy number, and presence of a transcript was determined in these as well as in two control plants. This is illustrated in the following Table 2 which provides a correlation of replicase gene copy number and resistance to CMV in R1 generation plants of segregating lines N/B 1-2 and 1-8 which were inoculated with 100 µg./ml and 500 µg/ml of Fny-CMV virus, respectively.

TABLE 2

Line	Systemic Symptom[a]	Viral Defective Replicase Presence[b]	Copy Number[c]	Resistance Gene Transcript[d]
1-2	-	-	2	ND
	+	+	0 or 1	ND
1-8	-	-	3 to 4	+
	+	+	3 to 4	-
N/N[f]	-[g]	-	0	-
	+[h]	+	0	-

[a] three symptomatic (susceptible) and three symptomless (resistant) plants were examined in lines N/B 1-2 and 1-8;

[b] the presence of viral RNA in uninoculated leaves was determined by dot blot hybridization [see Palukaitis, Methods for Plant Molecular Biology, Academic Press (New York), pgs 487-506 (1988)] using a Fny-CMV cDNA probe;

[c] the number of defective replicase gene copies per haploid gene was determined by Southern blot hybridization analysis of genomic DNA isolated from each plant using the 35S CaMV promoter as a probe.

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These genomic DNAs were digested with either HindIII/EcoRI which liberated a 2.75 kb fragment, *PstI* which liberated a 4.1 kb fragment as well as higher molecular weight fragments due to incomplete digestion of methylated DNA, and HindIII which liberated fragments of various molecular weights depending upon the site of insertion of the modified replicase gene in the genome;

[d] total RNA was isolated from each plant, and 40 µg was run in a formaldehyde 1.2% agarose gel, transferred to Genescreen Plus (DuPont) and hybridized using the NOS terminator sequence as a probe;

[f] the NN control line was *Nicotiana tabacum* cv. Turkish Samsun NN;

[g] uninoculated plant; and

[h] inoculated plant 100 µg/ml Fny-CMV.

Virus, as depicted in Table 2, was shown to be present only in those plants showing systemic symptoms. The copy number in the susceptible N/B 1-2 plants were 0 or 1 per haploid genome, whereas the resistant N/B 1-2 plants each contained 2 copies per haploid genome. This indicated that a copy of the gene is necessary but does not assure resistance, and the presence of multiple copies of the gene does not guarantee that a particular plant will be resistant. This is substantiated by analysis of the N/B 1-8 resistant and susceptible plants which showed that they all have incorporated three to four copies per haploid genome (Table 2). Furthermore, most of these insertions, whether from resistant or susceptible plants, appear to be in similar locations within the genome. Northern blot analyses of these susceptible and resistant N/B 1-8 plants showed that a transcript from this modified RNA-2 Fny-CMV RNA 2 was only present in the resistant plants (Table 2). These results indicate that resistance is engendered by the presence of the gene sequence in the tobacco genome and by its active transcription.

Many of the resistant plants have chlorotic lesions on the inoculated leaves, however, the level of viral RNA in the inoculated leaves of resistant plants is either very low or not detectable by dot-blot hybridization as compared to non-resistant plants, and in addition, viral RNA has not been detected by dot-blot hybridization in uninoculated leaves of resistant plants. These observations suggest that resistance is due to an inhibition of viral replication at the site of

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infection. Judging from the the spreading symptoms seen on CMV-inoculated leaves, some virus may move from cell to cell, but it does not invade or survive in the vascular system of the plant.

In conclusion, the data reported herein clearly show that transgenic tobacco plants containing a modified CMV-replicase gene are highly resistant to infection by CMV. Furthermore, the level of CMV replicase mediated resistance is maintained at an inoculum dose of 500 µg/ml which is 10-fold higher than that previously shown for CMV coat protein mediated protection [see Bio/Technology 6:549 (1988)]. The data also show that transgenic tobacco plants transformed with a 54-kDa gene encoded in the read through of a replicase protein also exhibit TMV resistance. In both cases described herein, replicase sequences are used for the plant transformation. Although the two systems exemplified herein, both of which involve transformation with viral replicase sequences, may operate by different mechanisms, it appears that this replicase-mediated resistance is generic and applicable to other plant and animal RNA viruses.

Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail ourselves of such changes and modifications which may be made for adapting the present invention to various usages and conditions. Accordingly, such changes and modifications are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims. The terms and expressions which have been employed in the foregoing specification are used therein as terms of description and not of limitation, and thus there is no intention, in the use of such terms and expressions, of excluding equivalents of the features shown and described, or portions thereof, it being recognized that the scope of the invention is defined and limited only by the claims which follow.

Among such modifications are, for example, the substitution of plant transformation vectors other than those specified in the examples

above. For example, vectors which are within the range of substitutes or equivalents are those such as pBIN19, pBI101, pRok1, pAGS135, pARC12, PGA470, pRAL3940, and pCT1T3, among others. Although the present invention has been exemplified with TMV and CMV, other plant viruses such as, alfalfa mosaic, members of the potexvirus, bromovirus, potyvirus and luteovirus groups which also contain viral replicase regions within their genomes are also encompassed by the present invention, as are the host plants transformed with genetic sequences related to the replicase portions of these viruses. Since it is known that similarities in sequences exist between the replicase (polymerase) regions of RNAs of many "unrelated" plant viruses [see for example, N. Habili et al., Nucleic Acids Research 17:9543 (1989)], including similarities between certain plant and animal RNA viruses, these are properly considered to be equivalents and therefore encompassed by the scope of the present invention.

The listing of all nucleic acid and amino acid sequences contained in this disclosure are reproduced in the following Sequence Listing:

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Milton Zaitlin, Daniel Golemboski and George Lomonossoff

(ii) TITLE OF INVENTION: Induction Of Resistance To Virus Diseases By Transformation Of Plants With A Replicase Portion Of A Plant Virus Genome

(iii) NUMBER OF SEQUENCES: 8

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCAGGA

(2) INFORMATION FOR SEQ ID NO:2:

BNSDOCID: <WO 9325068A1>

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAAAGACUGG UGAUAUUUCU GAUAUG 26

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGUUGUUAA

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1425 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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GUCAAAGAUU GCAUAUUGGA UAUGUCUAAG UCUGUUGCUG CGCCUAAGGA   146
UCAAAUCAAA CCACUAAUAC CUAUGGUACG AACGGCGGCA GAAAUGCCAC   196
GCCAGACUGG ACUAUUGGAA AAUUUAGUGG CGAUGAUUAA AAGGAACUUU   246
AACGCACCCG AGUUGUCUGG CAUCAUUGAU AUUGAAAAUA CUGCAUCUUU   296
AGUUGUAGAU AAGUUUUUUG AUAGUUAUUU GCUUAAAGAA AAAAGAAAAC   346
CAAAUAAAAA UGUUUCUUUG UUCAGUAGAG AGUCUCUCAA UAGAUGGUUA   396
GAAAAGCAGG AACAGGUAAC AAUAGGCCAG CUCGCAGAUU UUGAUUUUGU   446
AGAUUUGCCA GCAGUUGAUC AGUACAGACA CAUGAUUAAA GCACAACCCA   496
AGCAAAAAUU GGACACUUCA AUCCAAACGG AGUACCCGGC UUUGCAGACG   546
AUUGUGUACC AUUCAAAAAA GAUCAAUGCA AUAUUUGGCC CGUUGUUUAG   596
UGAGCUUACU AGGCAAUUAC UGGACAGUGU UGAUUCGAGC AGAUUUUUGU   646
UUUUCACAAG AAAGACACCA GCGCAGAUUG AGGAUUUCUU CGGAGAUCUC   696
GACAGUCAUG UGCCGAUGGA UGUCUUGGAG CUGGAUAUAU CAAAAUACGA   746
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BNSDOCID: <WO 9325068A1>

CAAAUCUCAG AAUGAAUUCC ACUGUGCAGU AGAAUACGAG AUCUGGCGAA 796
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UUGCUGCAUG UUUGGCCUCG AUGCUUCCGA UGGAGAAAAU AAUCAAAGGA 996
GCCUUUUGCG GUGACGAUAG UCUGCUGUAC UUUCCAAAGG GUUGUGAGUU 1046
UCCGGAUGUG CAACACUCCG CGAAUCUUAU GUGGAAUUUU GAAGCAAAAC 1096
UGUUUAAAAA ACAGUAUGGA UACUUUUGCG GAAGAUAUGU AAUACAUCAC 1146
GACAGAGGAU GCAUUGUGUA UUACGAUCCC CUAAAGUUGA UCUCGAAACU 1196
UGGUGCUAAA CACAUCAAGG AUUGGGAACA CUUGGAGGAG UUCAGAAGGU 1246
CUCUUUGUGA UGUUGCUGUU UCGUUGAACA AUUGUGCGUA UUACACACAG 1296
UUGGACGACG CUGUAUGGGA GGUUCAUAAG ACCGCCCCUC CAGGUUCGUU 1346
UGUUUAUAAA AGUCUGGUGA AGUAUUUGUC UGAUAAAGUU CUUUUUAGAA 1396
GUUUGUUUAU AGAUGGCUCU AGUUGUUAA 1425

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 132 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AAU ACC AUC GUC ACC AUG GCU GAG UUU GCC UGG UGU UAU GAC 42
ACC GAC CAA UUC GAA AAG CUU UUA UUC UCA GGC GAU GAU UCU 84
CUA GGA UUU UCA CUG CUU CCC CCU GUU GGU GAC CCG AGU AAA 126
UUC ACA 132

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 amino acids
(B) TYPE: peptide
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

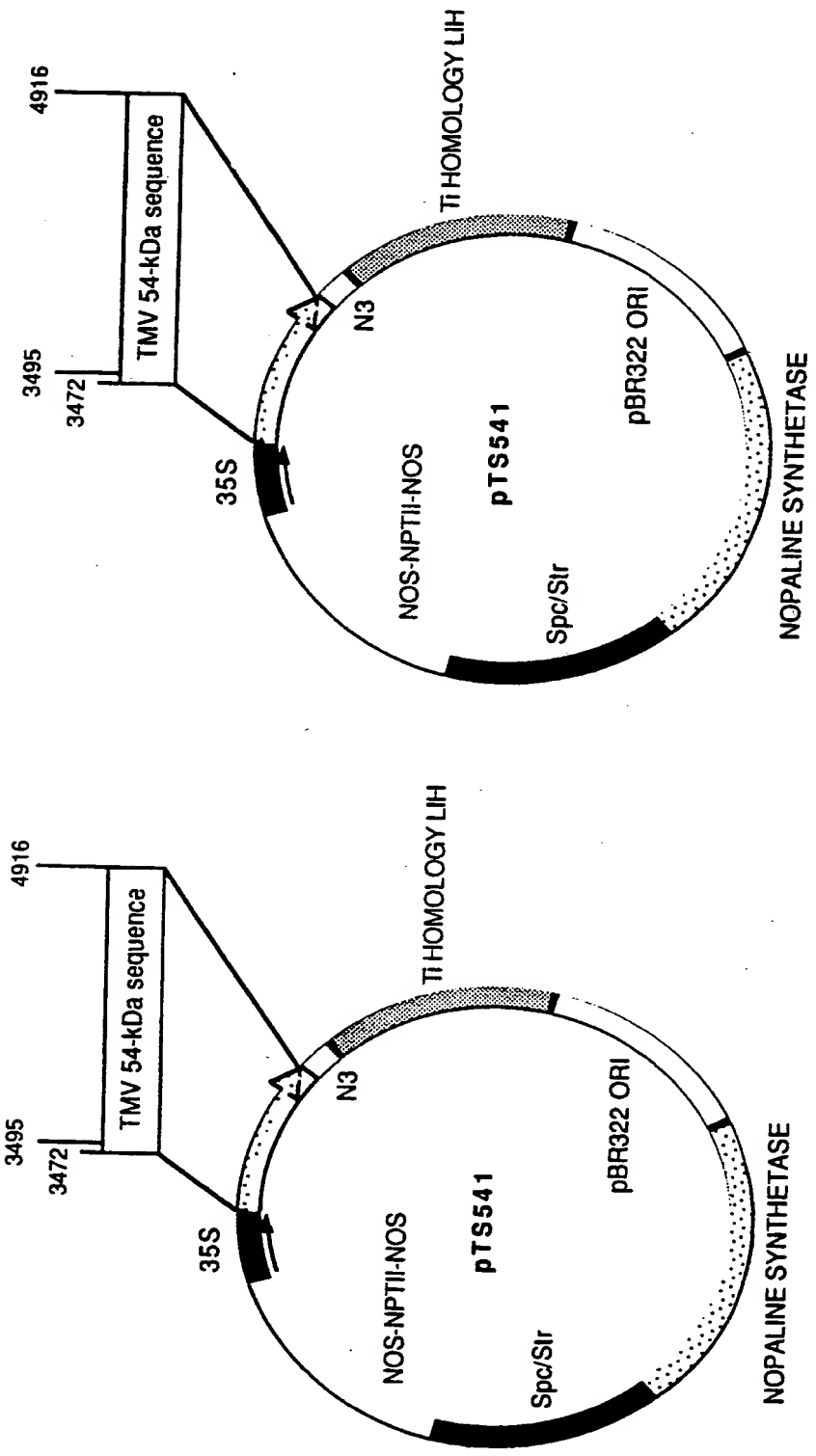
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Asn Thr Ile Val Thr Met Ala Glu Phe Ala Trp Cys Tyr Asp Thr
5 10 15
Asp Gln Phe Glu Lys Leu Leu Phe Ser Gly Asp Asp Ser Leu Gly
20 25 30 Phe
Ser Leu Leu Pro Pro Val Gly Asp Pro Ser Lys Phe Thr
35 40

(2) INFORMATION FOR SEQ ID NO:7:

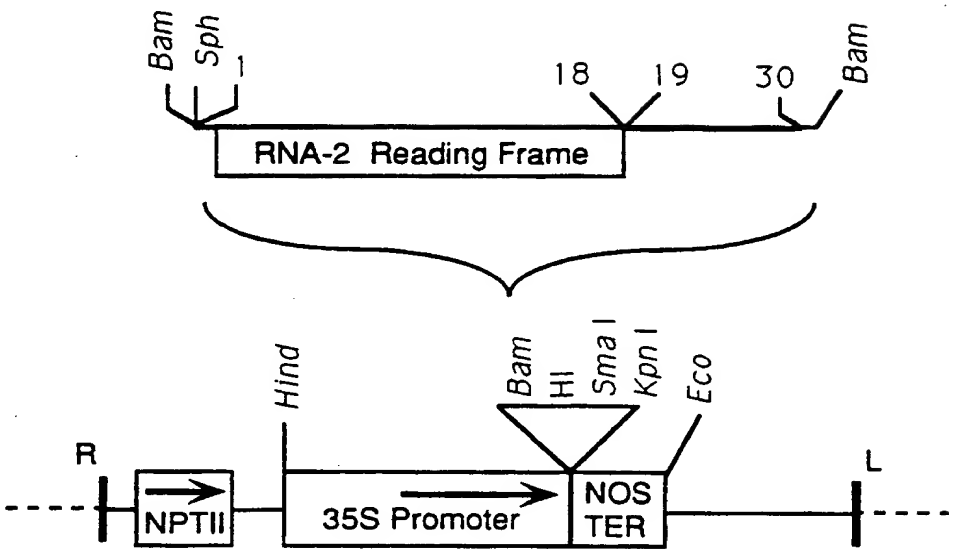
BNSDOCID: <WO 9325068A1>

FIGURE 1



BNSDOCID: <WO 9325068A1>

FIGURE 2



BNSDOCID: <WO 9325068A1>

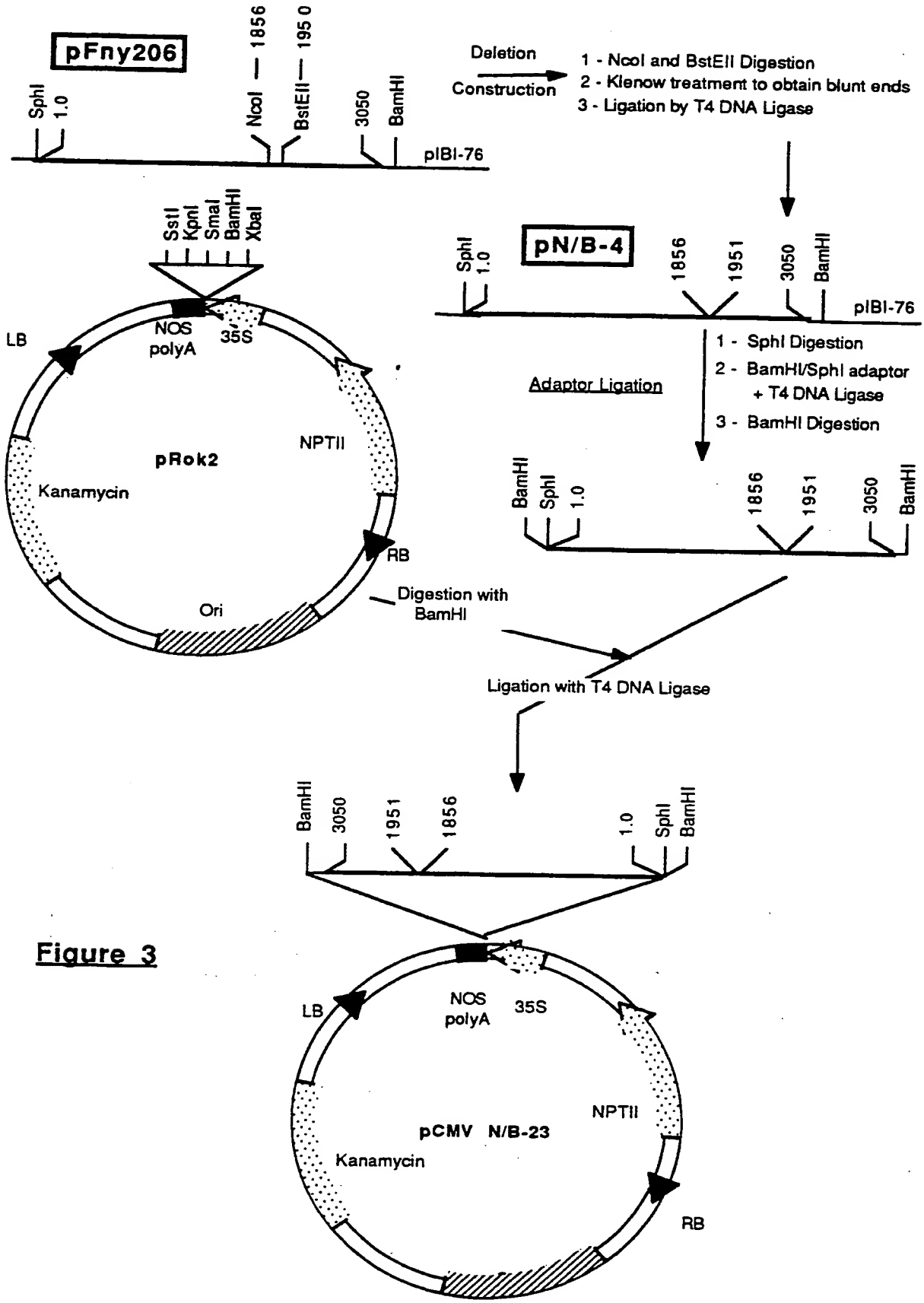


Figure 3

BNSDOCID: <WO 9325068A1>

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/05331

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :A01H 4/00; C12N 15/40, 15/82
US CL :800/205; 435/172.3
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 240.4, 948, 172.3; 536/23.72; 935/64; 800/205

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings National Academy of Science, USA, volume 79, issued October 1982, Goelet et al., "Nucleotide sequence of tobacco mosaic virus RNA", pages 5818-5822, see the entire document.	1-4
X	Virology, volume 145, issued 1985, Sulzinski et al., "Replication of tobacco mosaic virus. VIII. Characterization of a third subgenomic TMV RNA", pages 132-140, see the entire document.	2-4
Y		1-4

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
27 JULY 1993

Date of mailing of the international search report
26 AUG 1993

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Form PCT/ISA/210 (second sheet)(July 1992)*

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/US93/05331

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings National Academy of Science, USA, volume 86, issued September 1989, Powell et al., "Protection against tobacco mosaic virus antisense RNA", pages 6949-6952, see the entire document.	1-4
Y	Virology, volume 164(1), issued May 1988, van Dun et al., "Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not cross-protect against alfalfa mosaic virus infection", pages 383-389, see the entire document.	1-4
Y	Science, volume 232, issued 09 May 1986, Abel et al., "Delay of disease development in transgenic plants that express the tobacco mosaic virus coat protein gene", pages 738-743, see the entire document.	1-4

BNSDOCID: <WO 9325068A1>

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

Chemical Abstracts Online (File Biosis 1969-1993); USPTO Automated Patent System (File USPAT 1971-1993).
Search terms:

replicase
plant
virus
cucumber mosaic virus
tobacco mosaic virus

BNSDOCID: <WO 9325068A1>

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